PAPER NO. 10

QUINONES AND SULFHYDRYL-DEPENDENT IMMUNOTOXICITY

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INTRODUCTION

The work to be described is based on the use of immunologic models to understand mechanisms of chemical-induced toxicity to the lymphoreticular system. We sought to determine structure activity relationships through the study of target cell populations, the identification of toxic metabolites, and the analysis of factors which modulate toxicity.

SULFHYDRYL GROUPS AND IMMUNOTOXICITY

From the literature, it is known that membrane-penetrating sulfhydryl (SH) reagents such as N-ethylmaleimide (NEM) and cytochalasin A are more effective and specific for suppression of cell functions requiring cell surface receptor modulation (Edelman, 1976) and/or subtle shape changes than are SH reagents relatively impermeable to the cell membrane, including 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and p-chloromercuribenzene sulfonate (PCMBS). Some of these susceptible cell functions include phagocytosis (and exocytosis) (Elferink and Riemersa, 1980; Mazur and Williamson, 1977; Giordano and Lichtman, 1973; Tsan et al., 1976), blastogenesis (Chaplin and Wedner, 1978), and cell-mediated cytotoxicity (Cerottini and Brunner, 1972; Ralph and Nakoinz, 1980). Accordingly, intracellular SH groups may play a more important role than SH groups associated with ectoenzymes such as ATPases, nucleotide cyclases, and proteases which are also implicated in the regulation of these processes.

Suppression of blastogenesis by SH reagents does not involve changes in lectin-binding to the cell surface (Chaplin and Wedner, 1978; Greene et al., 1976; Berlin and Ukena, 1972; Sachs et al.,

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1973) and studies have repeatedly demonstrated that inhibition of phagocytosis and blastogenesis occurs at concentrations of disruptive agents which do not result in intracellular decreases in reduced glutathione (Chaplin and Wedner, 1978; Lagunoff and Wan, 1979), energy production (Mazur and Williamson, 1977; Lagunoff and Wan, 1979; Chakravarty and Echetebu, 1978; Pfeifer and Irons, 1981) or loss of membrane integrity (Elferink and Riemersa, 1980; Pfeifer and Irons, 1981). It is also known that colchicine must enter the cell to affect cell surface receptor movement (Aubin et al., 1975). Work from our laboratory suggests that particularly reactive SH groups on microtubules represent an intracellular target for SH alkylating agents like NEM and cytochalasin A which contain α,β-unsaturated carbonyl groups. We suggest that the benzene metabolite, p-benzoquinone (p-BQ), produces its immunotoxic effects via the same mechanism (Pfeifer and Irons, 1981; Irons et al., 1981; Pfeifer and Irons, 1982).

CELL-CELL INTERACTIONS AND THE CYTOSKELETON

Lectin-induced blastogenesis, as well as the development of immune responses, is dependent upon cell-cell interactions (Figure 1). Therefore, cell density in culture will influence these responses. In addition, non-lymphoid accessory cells, like macrophages, are also involved in the regulation of the final response (Rosenberg and Lipsky, 1981; Yoshinaga et al., 1972; McClain and Edelman, 1980; Suthanthiren et al., 1980). Cell-cell interactions are not only involved in the afferent arm of the immune response, but the appropriate apposition of cell surface structures, for example, specific receptors for sensitizing determinants and/or gene products of the major histocompatibility complex are also required for expression of lymphocyte-mediated cytotoxicity. This "matching" of cell surface structures occurs during the reversible, primary stage of effector cell/target cell interaction (Cerottini and Brunner, 1974; Pearson, 1978). As shown in Figure 2, both immune T cell cytotoxicity and antibody-dependent killing by a non-sensitized effector cell type (ADCC), the K cell, require modulation of cell surface structures and subtle changes in cell shape for lytic expression (Sanderson, 1981; Ryser and Vassalli, 1981). After exposure to appropriate activating stimuli, macrophages can also act as effector cells via either mechanism (Adams et al., 1982).

Other important cytoskeletal-dependent processes during the amplification of an immune response include phagocytosis, cell motility, and secretion. The cytoskeleton is also involved in the normal function of processes associated with a variety of other specialized cell systems particularly vulnerable to chemical toxicity. Some of these sensitive processes include secretion of hormones by endocrine organs, chemical transmission at the synaptic cleft, morphogenetic interactions during embryogenesis, and spermatogenesis.

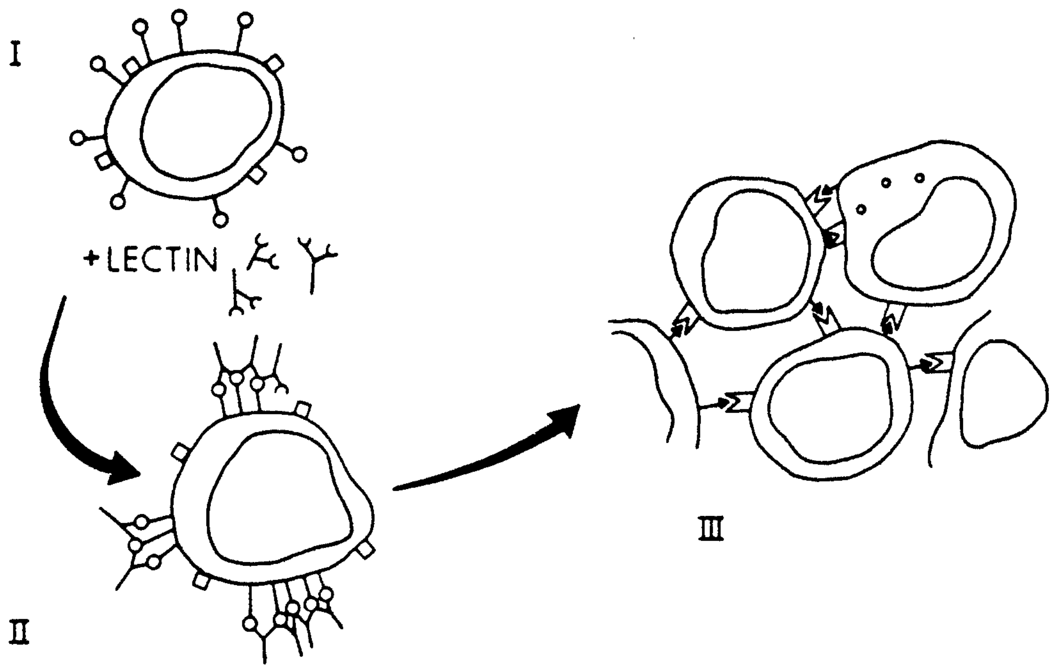


Figure 1. Stages of lectin-induced lymphocyte activation: I "Resting" lymphocyte population. II Transduction of the initiating signal - lectin dependent; cell-cell independent. III Induction of cell-cell communication - lectin independent; cell-cell dependent.

LYMPHOCYTE RESPONSE AND THE CYTOSKELETON

Cell growth and recognition in general are controlled through an assembly of interacting structures at or near the cell surface (Edelman, 1976). Cell surface receptors, glycoproteins in the case of lectin stimulation, extend through the lipid bilayer in random states of attachment with these structures known as microfilaments and microtubules, collectively referred to as the cytoskeleton. That the cytoskeleton modulates membrane receptor mobility can be shown by experiments measuring patch or cap formation after cross-linking cell surface receptors with a polyvalent ligand such as lectin or antibody (Figure 3). Microfilaments and energy are required for the capping phenomenon to occur, but microtubules appear restrictive to the process in that microtubule-disrupting agents will relieve the suppression of capping that occurs with excessive cross-linking of surface receptors. That benzene metabolites might act as microtubule-disrupting agents was suggested by experiments wherein µM concentrations of p-BQ appeared as

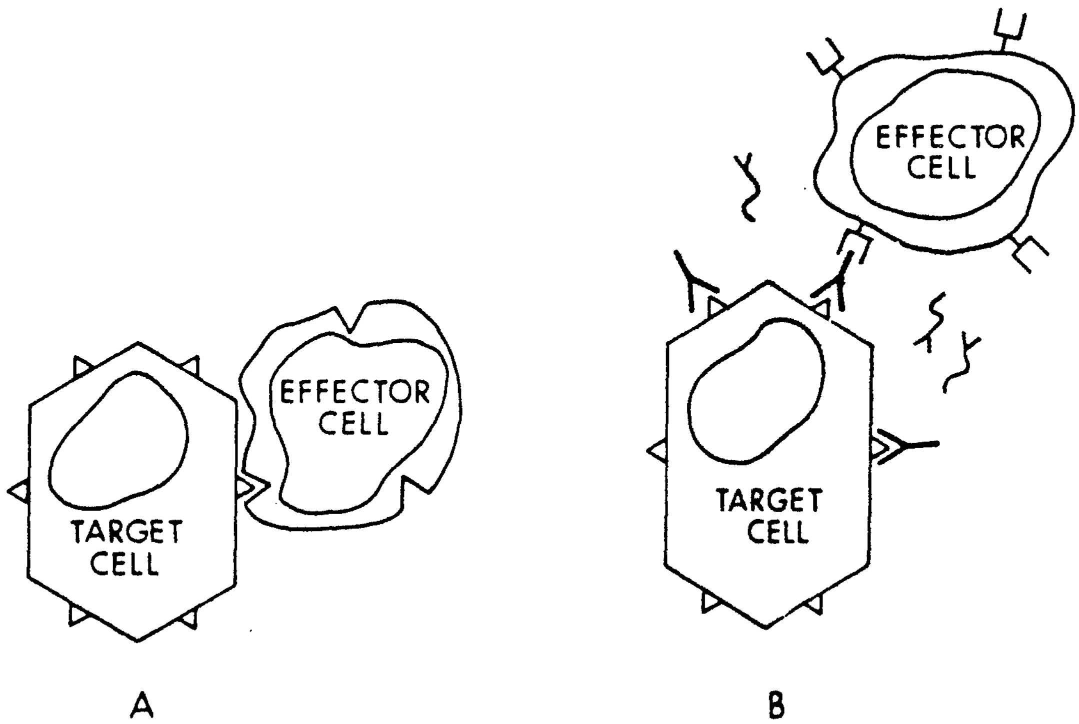


Figure 2. Cytotoxic lymphocyte effector cell-target cell interactions: A. Sensitized T effector cell demonstrates specific receptors in the outer membrane which react with determinants on the target cell. B. Null lymphoid effector cell implicated in antibody-dependent cellular cytotoxicity (ADCC), the K cell, demonstrates no specificity for the target cell, but is activated by the Fc portion of immunoglobulin G after antibody binds to specific target cell determinants.

effective as colchicine at enhancing capping of fluorescent antibody directed against lymphocyte cell surface immunoglobulin in the presence of saturating amounts of lectin (Irons et al.).

BENZENE IMMUNOTOXICITY: EFFECTS OF BENZENE METABOLITES ON A) LYMPHOCYTE FUNCTION AND B) MICROTUBULE ASSEMBLY

Chronic exposure to benzene results in a variety of blood dyscrasias including lymphocytopenia and pancytopenia both in animals and humans; an association with increased risk of leukemia has been made for human exposure (Snyder and Kocsis, 1975). Benzene is not itself considered to be the ultimate toxicant but is

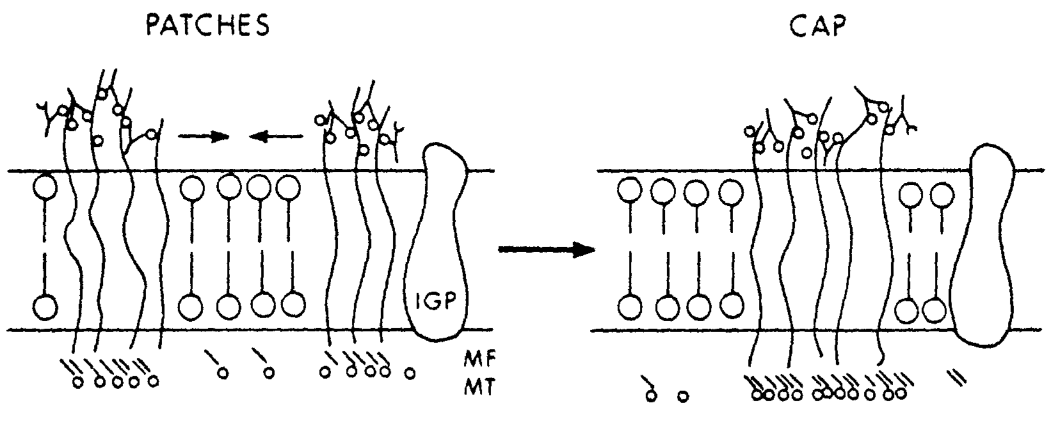


Figure 3. Cell surface receptor anchorage modulation: cell surface receptors span the lipid bilayer variably linked with submembranous microfilaments (MF) and microtubules (MT). PATCHES-crosslinking of receptors with a multivalent ligand such as lectin or antibody results in an alteration in the equilibrium of free versus anchored receptors. The result is an alteration of receptor mobility and effective distribution of receptor sites on the cell surface. CAP-with time, aggregated receptors sites coalesce to form a polar cap. IGP-intramembranous globular protein remains fixed despite alterations in linkage of adjacent receptor sites.

metabolized in vivo by the cytochrome P-450-dependent monooxygenase system to toxic intermediates (Figure 4). Previous work has consistently indicated a correlation between benzene-induced lymphocytopenia and immunotoxicity and the accumulation of hydroquinone (HQ) and catechol (CAT), but not phenol, in bone marrow and lymphoid tissues (Irons et al., 1981).

A) LYMPHOCYTE FUNCTION

The effect of HQ and p-BQ on phytohemagglutinin (PHA)-stimulated lymphocyte blastogenesis is shown in Table 1. Ficoll-purified rat spleen lymphocytes were preincubated with metabolite (usually 30 minutes) and then washed before addition of lectin to cultures. Preincubation with greater than μM concentrations of HQ results in suppression of blastogenesis whereas less than μM concentrations enhances [^{3}H] thymidine uptake to an amount at least twice that observed in control stimulated cultures. Enhancement was

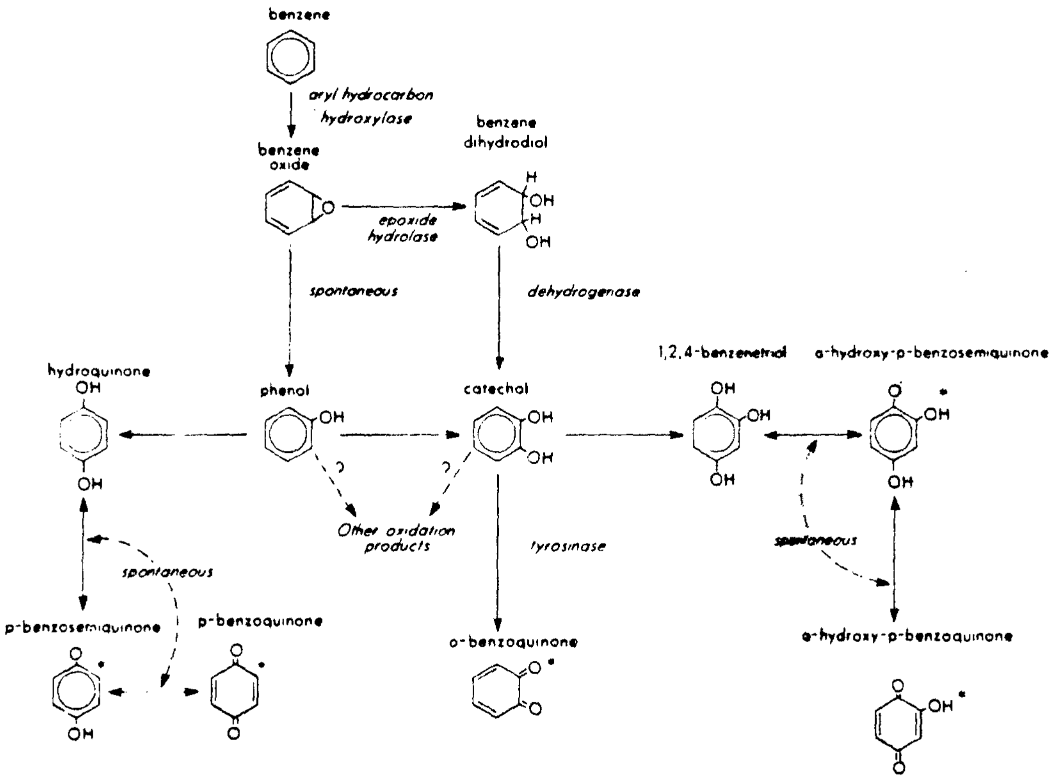


Figure 4. Schematic diagram of the metabolism of benzene demonstrating pathways resulting in the production of potentially reactive metabolites. Asterisks denote puntative or demonstrated alkylating activity toward intracellular nucleophiles.

generally found to be optimal at approximately 10^{-7} M, with stimulation returning to control levels at 10^{-9} M. Experiments wherein a range of lectin concentrations are used demonstrate that enhancement of blastogenesis at relatively higher concentrations of lectin (5.0 μg/ml) was accompanied by a reduction in response at lower concentrations of lectin (2.5 μg/ml) (Pfeifer and Irons, 1982). This type of result suggests that the stimulus threshold for growth response has changed for the responding cell population, allowing an additional response to occur at concentrations of lectin that were previously optimal. Cytoskeletal-disrupting agents such as colchicine and cytochalasin B, and other compounds reportedly giving rise to quinone intermediates such as diethylstilbestrol, have been reported to produce similar modifications of lymphocyte growth response (Yoshinaga et al., 1972; McClain and Edelman, 1980; Suthanthiren et al., 1980). These observations were the basis for developing an in vitro model for detecting chemical effects on differentiating rat bone marrow T cell precursors using flow cytometry to monitor

ontogenetic appearance of specific T cell surface markers (Pfeifer and Irons, 1982; Pfeifer et al.). At any lectin concentration, preincubation with 10^{-5} M HQ results in complete suppression of blastogenesis in the absence of cell death as determined by trypan blue exclusion or ATP production. All the polyhydroxy metabolites of benzene have a similar biphasic effect on PHA-stimulated blastogenesis with p-BQ the most suppressive, approximately twice as potent as HQ (Table 1), followed by 1,2,4-benzenetriol (BT) and CAT. Phenol is not toxic to cultured lymphocytes at any concentration examined.

TABLE 1. EFFECT OF HQ AND p-BQ ON PHA-STIMULATED RAT SPLEEN CELLS[a]

	Hydroquinone		p-Benzoquinone	
Concentration (x 10^{-7} M)	E/C Ratio	A.I.[b]	E/C Ratio	A.I.
4	2.07	++++	0.75	++++
6	1.90	++++	0.72	++++
8	1.56	++++	0.38	+++
10	1.36	++++	0.44	+++
20	0.32	+++	0.01	++
40	0.05	++	0.00	-
60	0.03	+	0.01	-
80	0.03	-	0.00	-
100	0.02	-	0.01	-

[a]Responses of Ficoll-purified cells pooled from F-344 male rats were assayed at optimal time points (48-72 hours after mitogen addition), cell (10^6 cells/ml) and lectin (5 μg/ml) concentrations. Values are expressed as the stimulation ratio of experimental to control (E/C) cpm. Results are calculated from the means of triplicate cultures wherein the S.D. of the mean did not vary more than 10%.

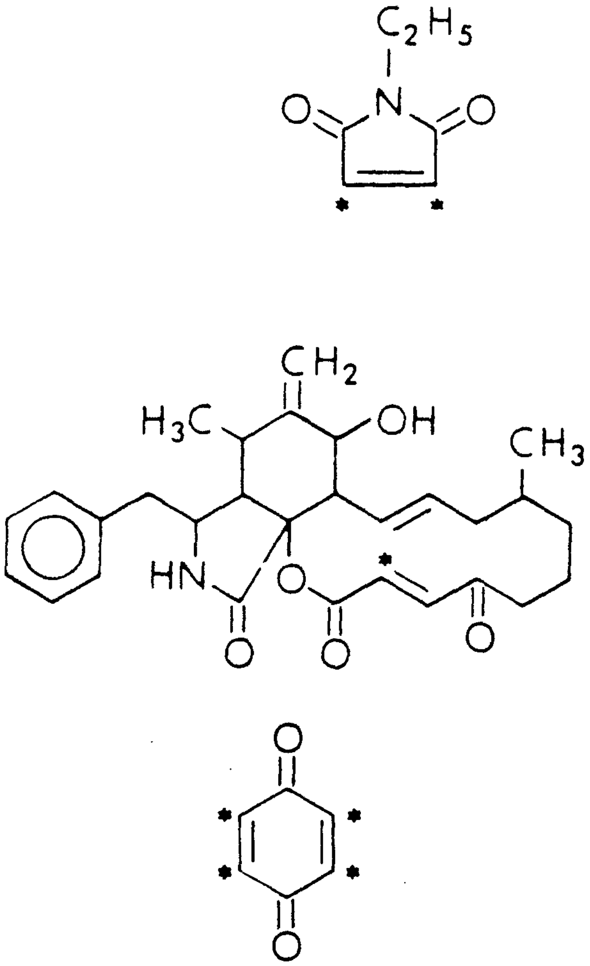
[b]Agglutination index (A.I.) indicates degrees of cell aggregation and blast transformation after exposure to PHA as observed by phase-contrast microscopy: +, appearance similar to cultures with no mitogen; ++ or +++, increasingly larger aggregates of cells, including blasts, and increased numbers of aggregates; ++++, appearance similar to cultures receiving no metabolite pretreatment; -, cells separate and equally spaced with no evidence of aggregation or blast transformation.

It was observed that suppression of lectin-stimulated agglutination occurs in parallel with suppression of blastogenesis (Table 1). Pretreatment with sublethal inhibitory concentrations (10^{-5} M) of HQ or NEM results in complete suppression of lectin-induced lymphocyte agglutination and blast transformation; in culture, the cells appear separate and equally spaced, demonstrating less cell-cell contact than that observed in unstimulated cultures. Although there is not uniform consensus on the importance of cell-cell interactions in the initiation of cell division, agglutination has been reported to be a a prerequisite for blastogenesis and certainly represents one of the earliest events associated with cell division (Wedner and Parker, 1976). Spectrophotometric quantitation of PHA-induced lymphocyte agglutination suggests that the increased adherence properties of lymphocytes occurring within minutes of exposure to lectin is inhibited concomitantly with blastogenesis by µM concentrations of membrane-penetrating SH alkylating agents; more interesting, the agglutination phenomenon is enhanced at the same low concentrations (<µM) of the agents which result in augmentation of blastogenesis as measured by [^{3}H] thymidine uptake several days later (Pfeifer and Luster, 1983).

Microtubules, important in the mediation of lectin-induced agglutination (Berlin and Ukena, 1972), have been suggested to be a requirement for transduction of the initiating signal for blastogenesis (Edelman, 1976; Greene et al., 1976; Gunther et al., 1976; Sherline and Munday, 1977; Wang et al., 1975). This does not necessarily require that the two events, signal transduction and agglutination, occur at the same point in the commitment to blastogenesis. The relationship of agglutination to blastogenesis may be explained by events happening somewhat later than mitogenic signal transduction.

The structures of NEM, cytochalasin A and p-BQ all feature a highly polarized, unsaturated carbon-carbon bond (carbonyl electron-withdrawing groups on either side) which is subject to attack by highly reactive SH groups acting as nucleophiles; a conjugate addition reaction (Figure 5). HQ, which theoretically autoxidizes to the p-BQ product, was compared to NEM for effects on cell function. After pretreatment of cells, both HQ and NEM produce a sublethal, concurrent inhibition of lymphocyte blastogenesis and agglutination at the same concentrations. The addition of a SH compound, dithiothreitol (DTT), to the incubation tube with either HQ or NEM protects against the inhibitory effects of both agents in a concentration-dependent manner (Figure 6). Similar effects on cultured cells have been noted in our laboratory for cytochalasin A and p- and o-aminophenol. The latter two compounds are aniline metabolites and are theoretically capable of oxidative conversion to benzoquinoneimine derivatives (Pfeifer and Irons, 1983). However, pretreatment with DTNB, a poorly penetrating SH reagent, failed to inhibit either agglutination or PHA mitogen response (Figure 6). These findings are consistent with the selectivity of HQ and NEM for SH groups relative to other nucleophilic groups. Although cysteine

affords complete protection, preincubation with mM lysine, serine, and imidazole at physiologic pH fail to protect against toxicity by these SH reagents. Therefore, it appears that 1) HQ suppresses blastogenic response by interacting with intracellular SH sites and 2) sublethal impairment of immune function by HQ is mimicked by a SH alkylating reagent, NEM.



N-Ethylmaleimide

Cytochalasin A

p-benzoquinone

Figure 5. Structures demonstrated to alkylate SH groups at physiologic pH via Michael addition. Asterisks denote carbons subject to nucleophilic attack.

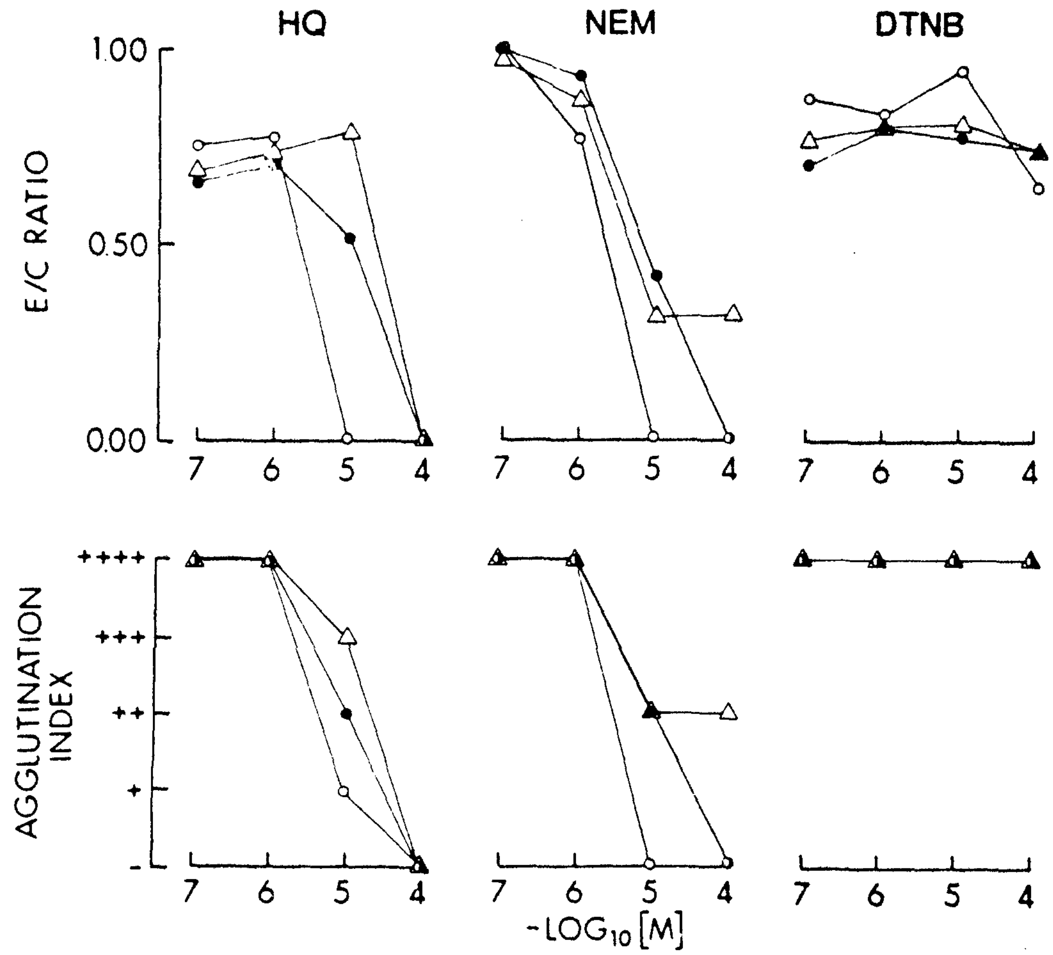


Figure 6. Protection against HQ or NEM inhibition of PHA-stimulated blastogenesis and agglutination in Ficoll-purified rat spleen lymphocytes by DTT. Comparisons of effects of NEM, a cell penetrating SH reagent, with DTNB, a poorly penetrating SH reagent. O, No DTT present during preincubation with HQ or SH reagents; Δ, 10^{-4} DTT present, ●, 10^{-5} M DTT present during preincubation. Results for blastogenesis expressed as stimulation ratio of experimental to control (E/C) cpm. Results are calculated from the means of triplicate cultures wherein the S.D. of the mean did not vary more than 10%. Agglutination index determined as for Table 1.

B) MICROTUBULE ASSEMBLY

The integrity of SH groups on tubulin is a functional requisite for stability. The GTP-binding site on tubulin has been well characterized and involves two out of approximately eleven titratable SH binding sites varying somewhat on the method of isolation. It is known that these two SH groups are statistically more reactive with SH alkylating agents which inhibit microtubule assembly than other SH sites on the tubulin molecule (Ikeda and Steiner, 1978; Kuriyama and Sakai, 1974; Mann et al., 1974; Mellon and Rebhun, 1976).

Cycle-purified rat brain tubulin was isolated to monitor the effects of benzene metabolites on in vitro activity. Temperature dependent tubulin polymerization was measured turbidimetrically at 350 nm following addition of p-BQ, HQ, NEM, or BT at various concentrations. Polymerization was inhibited by all of these agents in a concentration-dependent manner (Figure 7). The stoichiometry of the HQ:tubulin interaction suggests a small number of binding sites (Edelman, 1976; Elferink and Riemersa, 1980; Mazur and Williamson, 1977) are involved in the inhibition of this function (Irons et al., 1981). The linearity of the semilogarithmic plot of inhibition versus the concentration of p-BQ or NEM is characteristic of a first order reaction and suggests a direct reaction of p-BQ and NEM with tubulin to inhibit polymerization; colchicine demonstrates a similar linear plot. Minimally effective concentrations of p-BQ and NEM are the same, although differences in slope of the lines may reflect increased affinity for additional SH sites by NEM relative to p-BQ.

Inhibition of polymerization by HQ and BT is non-linear, indicating the kinetics of inhibition are complex. Other experiments suggest that this reflects the requirement for HQ and BT to be autoxidized to reactive quinone or quinone analogs before reaction with tubulin (Irons et al., 1981). Conversely, anaerobic conditions did not protect against the effects of NEM and p-BQ on tubulin. DTT, but not lysine or serine, protects against the effects of p-BQ and NEM on tubulin, analogous to experiments with whole cells. CAT has no effect on polymerization (5×10^{-4}M); however, if tyrosinase (10 μg/ml) is added to the system, as little as 10^{-5} M CAT results in complete loss of tubulin polymerization. Tyrosinase converts CAT to a highly reactive o-quinone directly via a two electron transfer, suggesting a direct effect of the quinone on tubulin.

HOW DOES THIS MECHANISM FIT IN WITH TRADITIONAL MODELS OF ACTIVATION?

It is known that modulation of intracellular cyclic nucleotides represents an important secondary signal for activation of gene derepression. In many pharmacological models, but especially in the

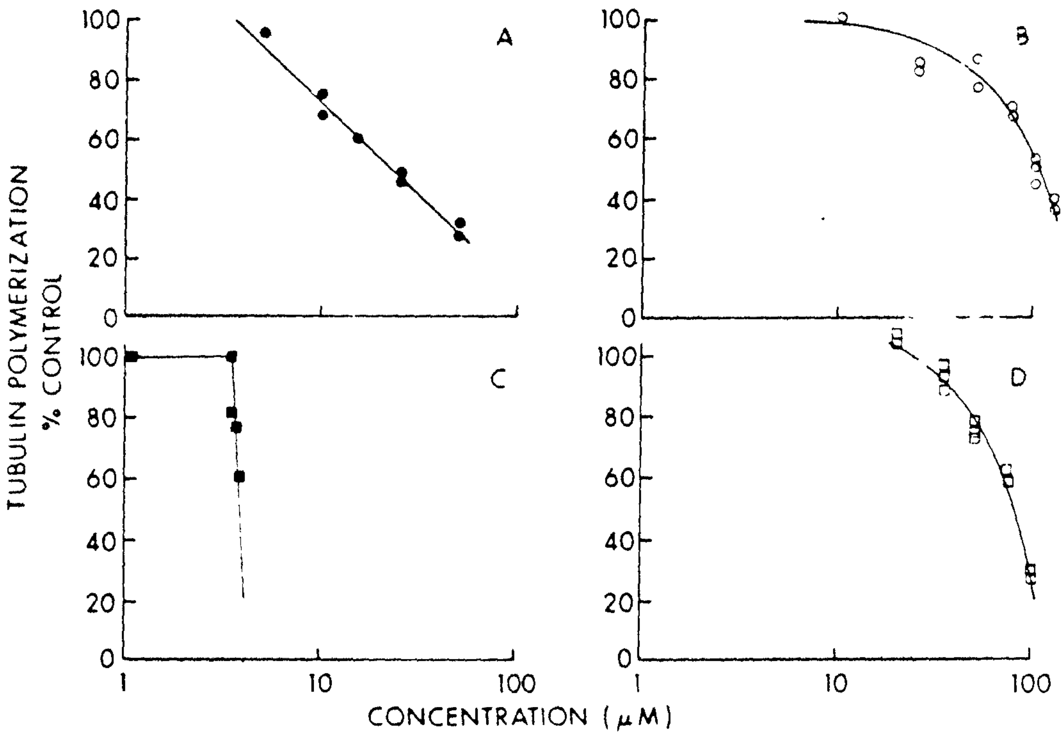


Figure 7. Log concentration-inhibition curves for a) p-BQ, b) HQ, c) NEM, and d) BT on cycle-purified rat brain tubulin polymerization or self-assembly in vitro. Temperature dependent polymerization was measured turbidmetrically at 350 nm after 10 min. incubation at 37°C. Inhibition calculated as percent of control.

area of immunoregulation, there is a satisfying correlation between changes in intracellular levels of these mediators and modulation of cell functions; net increases in cGMP result in enhancement of secretion, cell-mediated cytotoxicity, proliferation and differentiation of immune cells, while net increases in cAMP result in suppression of these functions (Ignarro, 1977; Gillespie, 1977; Strom and Carpenter, 1977; Henney et al., 1972; Lane, 1978; Strom et al., 1972; Hadden, 1977; Watson, 1977). However, it is also true that mobilization of extra- and intracellular Ca^{2+} stores and an intact cytoskeleton appear to be a common denominator for effective functional response to specific initiating signals in the same cellular models (Edelman, 1976; Cerottini and Brunner, 1972; Rosenberg and Lipsky, 1981; Yoshinaga et al., 1972; Sanderson, 1981; Ryser and Vassalli, 1981; Adams et al., 1982; Weissmann et al., 1981; Henson et al., 1981; Keller et al., 1981; Gale and Zighelboim, 1974). Recent evidence demonstrating the intimate association of calmodulin

(Ca^{2+} binding protein), membrane nucleotide cyclases and microtubules (Rasenick et al., 1981; Watanabe and West, 1982) suggests a transducing role for the cytoskeleton which might be susceptible to chemical/drug regulation. For example, microtubule-disrupting agents appear to increase cAMP response in stimulated cells, presumably by influencing the mobility of cell surface receptors and membrane adenylate cyclase activity (Greene et al., 1976; Rudolph et al., 1977).. Alternatively, direct cell-cell contact is a prerequisite for full functional expression of cell activation and might well represent a secondary locus for expression of injury to cytoskeletal structures.

Although we have not looked at the effects of NEM or p-BQ preincubation on other early events of lymphocyte activation such as RNA and protein synthesis, other investigators have demonstrated that colchicine, at concentrations that specifically inhibit DNA synthesis, also significantly inhibits these early biochemical events (Sherline and Mundy, 1977). Since preincubation of lymphocytes with µM NEM or p-BQ results in failure to undergo blast transformation, (Pfeifer and Irons, 1981; Irons et al., 1981; Pfeifer and Irons, 1982), we hypothesize a similar suppressive activity for membrane-penetrating SH alkylating agents, concomitant with inhibition of microtubule assembly. Significantly greater concentrations (0.1-1.0 mM) are required to inhibit plasma-membrane associated regulatory enzymes including guanylate cyclase (Haddox et al., 1978), or those involved in energy metabolism.

CONCLUSIONS

We suggest that early changes in cell agglutination after lectin binding require intact microtubules. These changes, like other early cell responses including increased Ca^{2+} accumulation and cGMP dependent protein kinase levels (Hadden, 1977), occur within minutes. The fact that hormonal and neurotransmitter agents that increase cGMP do so only in intact cells and that activation requires Ca^{2+} suggests an important role for microtubules and calmodulin in the activation of guanylate cyclase. Furthermore, the intimate interrelationship of calmodulin, membrane cyclases, microtubules and phosphodiesterases suggests that effects on one component would be expected to influence the function of the others and that none dominates the functional response of activated cells (Watanabe and West, 1982).

Particularly reactive SH groups on tubulin may constitute an intracellular target, important to normal growth control of the mammalian cell, which are uniquely sensitive to p- and o-quinone metabolites of immunotoxic xenobiotics or analogous resonance structures that possess SH-alkylating activity. Potency differences are probably related to both the efficiency with which oxidation of precursor molecules to reactive quinone structures occurs, and to the SH-reactivity of the quinone or substituted quinone.

The apparent sensitivity of microtubule assembly to SH-alkylating reagents suggests that the process may be susceptible to regulation by similarly reactive endogenous molecules under normal physiologic circumstances. For example, recent work quantitating PHA-induced lymphocyte agglutination spectrophotometrically suggests that the catechol estrogen metabolites are the most potent in suppressing this function, although the parent compound, 17β-estradiol, demonstrates little or no activity (Pfeifer and Luster, 1983).

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ACKNOWLEDGEMENTS

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